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Ethidium Bromide MIC Screening for Enhanced Efflux Pump Gene Expression or Efflux Activity in *Staphylococcus aureus*▽

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Multidrug resistance efflux pumps contribute to antimicrobial and biocide resistance in *Staphylococcus aureus*. The detection of strains capable of efflux is time-consuming and labor-intensive using currently available techniques. A simple and inexpensive method to identify such strains is needed. Ethidium bromide is a substrate for all but one of the characterized *S. aureus* multidrug-resistant (MDR) efflux pumps (NorC), leading us to examine the utility of simple broth microtiter MIC determinations using this compound in identifying efflux-proficient strains. Quantitative reverse transcription-PCR identified the increased expression of one or more MDR efflux pump genes in 151/309 clinical strains (49%). Ethidium bromide MIC testing was insensitive (48%) but specific (92%) in identifying strains with gene overexpression, but it was highly sensitive (95%) and specific (99%) in identifying strains capable of ethidium efflux. The increased expression of *norA* with or without other genes was most commonly associated with efflux, and in the majority of cases that efflux was inhibited by reserpine. Ethidium bromide MIC testing is a simple and straightforward method to identify effluxing strains and can provide accurate predictions of efflux prevalence in large strain sets in a short period of time.

Efflux is an important mechanism of antimicrobial agent and biocide resistance in *Staphylococcus aureus*. The efflux process is mediated by membrane-based proteins capable of transporting a single class or several structurally distinct compounds, using either an ion gradient (commonly H^+) or ATP cleavage to provide the required energy (18). Proteins that transport two or more antimicrobials or biocides are called multidrug-resistant (MDR) efflux pumps. MDR efflux pump activity can predispose *S. aureus* to the acquisition of high-level target-based resistance mechanisms, with a relevant example being the appearance of topoisomerase mutations following exposure to fluoroquinolone substrates (14). Substrate exposure can increase efflux pump gene expression by their binding to and altering of the activity of regulatory proteins or the selection of regulatory mutations (4, 8, 10). The increased expression of MDR pumps leading to increased efflux activity may compromise therapy with substrate compounds, especially in cases where drug delivery may be marginal. At institutions where substrate quaternary biocides are used in cleaning, increased expression also may favor environmental survival and later acquisition by patients.

Using quantitative reverse transcription-PCR (qRT-PCR), we found that the increased expression of one or more chromosomal MDR efflux pump genes was present in half of all *S. aureus* bloodstream isolates recovered in 2005 from Detroit-area patients (2, 3). However, this approach requires specialized instrumentation and is expensive in terms of both supplies and hands-on time. In addition, it does not provide phenotypic information with respect to efflux. A simple and inexpensive method to identify *S. aureus* isolates having an efflux phenotype would be useful for determining the prevalence of increased MDR efflux pump activity among clinical strains from any source. MIC determinations in the presence and absence of efflux pump inhibitors is one approach, but we have found this to be insensitive among clinical strains (3). Fluorometric or radiometric assays of efflux activity would provide accurate prevalence data but suffer from being time-consuming and labor-intensive. An agar-based approach using ethidium bromide (EB), which is a known substrate for all but one of the characterized *S. aureus* MDR efflux pumps (NorC), has been described and is technically straightforward but has not been evaluated using large numbers of clinical isolates (5, 15). In addition, this method requires a series of plates containing various EB concentrations, and the interpretation of results may be affected by operator subjectivity. Because of these limitations, we evaluated the utility of simple microtiter EB MIC determinations as an inexpensive screening procedure for identifying strains having increased expression of MDR efflux pump genes or an efflux phenotype among a large collection of clinical *S. aureus* isolates obtained from several geographic locales.

MATERIALS AND METHODS

Strains. Bloodstream isolates of *S. aureus* (one per patient) were obtained from medical centers in Boston, Detroit, Omaha, Houston, and Freiberg, Germany ($N = 256$). In addition, non-bloodstream isolates from a variety of clinical sources (also one per patient) were obtained from centers in San Francisco ($N = 53$).

Microbiologic procedures. Reagents and media were obtained from Sigma Chemical Co., St. Louis, MO, and BD Biosciences, Sparks, MD, respectively. *S. aureus* SH1000 was used as a control strain for efflux assays and qRT-PCR (6). EB MICs were determined in quadruplicate using a microdilution procedure according to CLSI guidelines (1). EB efflux by selected strains was determined fluorometrically as described previously, with data presented as percent efflux (means ± standard deviations) during the 5-min time course of the experiment

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TABLE 1. Primers and TaqMan probes[a]

Gene[c]	Primer sequence	Reporter-probe sequence-quencher[b]
mdeA		
Fwd	CTTTCAGGTTACCTTGTTGAATATTTAAAC	JOE-AGATCGCTTTTCCATGTTGTCGCTCCAATT-BHQ1
Rev	ATCAATAGGTACTTTAATTGTAGTTCCAAC	
mepA		
Fwd	ATGGTATAGGTTTCTTGTTTACTGGTATG	JOE-AATTGTAGCACCACGACCTTGCCCAGTC-BHQ1
Rev	AATGATAATTGCACCTTGTAAAATGGC	
norA		
Fwd	TTTGTTTTCAGTGTCAGAATTTATGTTTG	6FAM-AGGCATAACCATACCAGCACTCATACCACC-BHQ1
Rev	GGCTTGGTGAAATATCAGCTATTAAAC	
norB		
Fwd	GTAATGGTACTAATTATGATTCGTGTTGG	TAMRA-ACTTCAAACACTCGGATGCAAGAAACCAATGT-BHQ2
Rev	CTGGCAAGAAAGTTAATGAAATGAGAC	
norC		
Fwd	CAGGCAGGATACTTATCAATTAC	6FAM-CATGGTGTTATTGATGATTCGAGTTGGTG-BHQ1
Rev	ATACCAATGACCACAATGAATG	
sdrM		
Fwd	CAACATGGCATTGGTTATTCTAC	6FAM-ACCGATTGCGATCATTGCCATTAT-BHQ1
Rev	ACAGCTGTTGGTTTAATAAAGC	
sepA		
Fwd	GAAGTATGTACGATAACCTATTATATTATGGC	JOE-TCTTTGGGTACTTCATCATATTTGCAGTCGAGC-BHQ1
Rev	AAAGTCGCGCCTCTAAAATATGC	
16S rRNA		
Fwd	CCAGCAGCCGCGGTAAT	CY5-CGTAGGTGGCAAGCGTTATCCGGA-BHQ3
Rev	CGCGCTTTACGCCCAATA	
mecA		
Fwd	AACCGAAGATAAAAAAGAACC	Not applicable
Rev	GTCCGTAACCTGAATCAGC	
qacA and *qacB*		
Fwd	GGTGCTTTAATAATGCC	Not applicable
Rev	CCAGTCCAATCATGCCTGC	

[a] Sequences are provided in 5′-to-3′ orientation.
[b] JOE, 6-carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein; 6FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; BHQ, black hole quencher. BHQ1, BHQ2, and BHQ3 maximal quenching wavelengths were 534, 579, and 672 nm, respectively.
[c] Fwd, forward; Rev, reverse.

(12). Efflux of ≥20% (2.5-fold that of *S. aureus* SH1000; EB MIC, 6.25 μg/ml; 7.9% efflux) was considered significant and representative of an efflux phenotype. Strains selected for efflux assays included all those with an EB MIC of ≥25 μg/ml ($N = 84$) and the same number of strains with an EB MIC of ≤12.5 μg/ml. The effect of reserpine (20 μg/ml) on strains having an efflux phenotype also was determined.

Gene expression. Seven genes on the *S. aureus* chromosome encode MDR efflux pumps, including *mdeA*, *mepA*, *norABC*, *sdrM*, and *sepA* (7, 11, 13, 16, 20–22). All of the encoded pumps, as well as QacA/B, which are plasmid-encoded MDR pumps, are capable of EB transport (5). The presence of *qacA* or *qacB* was detected using routine PCR and primers designed based on the sequence of *qacA* (GenBank accession no. X56628) that also had complete homology with *qacB* (Table 1) (17). For chromosomal genes, RNA was recovered and purified as described previously (2). qRT-PCR was used to quantify the expression of MDR pump genes by employing the Quantitect multiplex RT-PCR kit and instructions provided by the manufacturer (Qiagen, Inc., Valencia, CA). Beacon Designer 7.80 (Premier Biosoft International, Palo Alto, CA) was used to design TaqMan probes and primers, which were obtained commercially (Eurofins MWG/Operon, Huntsville, AL). To minimize false-negative qRT-PCR data from clinical strains, primer and probe sequences were optimized for the least number of mismatches using 13 *S. aureus* genome sequences available at http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi. Probes were labeled as described in Table 1, and qRT-PCR was performed in triplicate using an ABI 7500 fast real-time PCR system with parameters of 45°C for 20 min, 95°C for 15 min, and 40 cycles of 1 min at 94°C and 1 min at 55°C (Applied Biosystems, Foster City, CA). Positive controls for each gene were included, and 16S rRNA was used as the endogenous control. The comparative threshold cycle method was used to determine relative gene expression compared to that of *S. aureus* SH1000, in which the expression of each gene was considered to be 1.0. Values of 4.0 or greater were considered indicative of increased gene expression. Multiplex reactions included 16S rRNA primers and probe combined with those of *mdeA* and *norA*; *mepA*, *norB*, and *norC*; or *sdrM* and *sepA*.

Data analysis. Data were analyzed in two ways. Associations between an EB MIC of ≥25 μg/ml and either the increased expression of at least one MDR efflux pump gene or the presence of an efflux phenotype were defined as described in Table 2. Sensitivity, specificity, and positive and negative predictive values (PPV and NPV, respectively) were calculated using algorithms available at

TABLE 2. Definitions employed in this study

Result	EB MIC (μg/ml)	Expression of MDR efflux pump genes	Efflux phenotype (% efflux)
True positive	≥25	Increased expression of ≥1 gene	≥20
False positive	≥25	No increase	<20
True negative	≤12.5	No increase	<20
False negative	≤12.5	Increased expression of ≥1 gene	≥20

TABLE 3. EB MIC (in μg/ml) and increased pump gene expression[a]

Expression	MIC_{50}/MIC_{90} (range)	No. (%) of strains by EB MIC		Total
		≥25	≤12.5	
Increased expression of ≥1 gene by qRT-PCR	25/50 (3.13–≥100)	72 (47.7; TP)	79 (52.3; FN)	151
No increased expression	6.25/12.5 (1.56–50)	12 (7.6; FP)	146 (92.4; TN)	158
Total		84	225	309

[a] TP, true positive; FP, false positive; TN, true negative; FN, false negative.

http://www.neoweb.org.uk/Additions/predict.htm. Fluorometric efflux data were compared using SigmaStat 3.1 (Systat Software, Inc., Chicago, IL).

RESULTS AND DISCUSSION

Strain characteristics. Methicillin-resistant strains (MRSA) comprised 63% of isolates, with significant geographic variability. Among U.S. strains the proportion that were MRSA ranged from 55% (San Francisco) to 76% (Houston), which is indicative of the known heavy burden of such strains in many regions of the country (9). Only 1/16 German strains were MRSA (6.3%), which is consistent with the relatively low prevalence of this trait among German *S. aureus* isolates (19%) (www.rivm.nl/earss). These data indicate that the strains included in this study are typical of what might be encountered clinically for the geographic locale of origin.

qRT-PCR and detection of *qacA* and *qacB*. The increased expression of one or more MDR efflux pump genes or the presence of *qacA* and *qacB*, encoding the QacA/B MDR efflux pumps, was identified in 151 of the 309 tested strains (48.9%). In the majority of cases these strains overexpressed only one gene or possessed *qacA* and *qacB* without the increased expression of any of the other genes included in our analysis (82.1%; data not shown). Among those overexpressing a single gene, *norA* was most common (43%), followed by *norB* (23.2%) and *mepA* (9.9%). PCR established the presence of *qacA* and *qacB* in 3.3% of strains, and various combinations of genes were overexpressed in 17.9% of strains. Similar results were observed in our earlier evaluation of bloodstream isolates from Detroit-area patients. In that study, 63% of pump gene overexpressers demonstrated the increased expression of a single gene, among which *norA* predominated (32%) (2, 3). These data suggest that a common mode of regulation, such as global regulatory proteins, for two or more of the MDR pump genes included in our analysis is unlikely. Alternatively, the increased expression of more than one MDR pump gene may have deleterious physiologic consequences for the organism and thus is uncommon.

EB MIC and gene expression analyses. The relationship between the EB MIC and increased MDR pump gene expression, or the lack thereof, is provided in Table 3. From these data it is clear that the absence of *qacA* and *qacB* and/or the increased expression of any of the pump genes included in our analysis is infrequently associated with an EB MIC of ≥25 μg/ml (false positives, 7.6% of strains), whereas the MIC was at least 25 μg/ml slightly less than half the time (true positives, 47.7%) for strains with the increased expression of at least one evaluated pump gene. The highest MICs were observed for those possessing *qacA* and *qacB*, where values of ≥100 μg/ml were found for all such strains ($N = 5$).

The sensitivity, specificity, PPV, and NPV at an EB MIC of ≥25 μg/ml in predicting the presence of the increased expression of at least one MDR efflux pump gene were 47.7, 92.4, 85.7, and 64.9%, respectively. The low sensitivity and NPV are the results of the high numbers of false negatives; the EB MIC for more than half of the pump gene-overexpressing strains was ≤12.5 μg/ml. It is known that increased gene expression in *S. aureus* may not correlate well with increased quantities of the encoded protein, and it is likely that the low sensitivity and negative predictive values are related in large part to this fact (19). The high specificity value indicates that if the EB MIC is ≤12.5 μg/ml, then increased pump gene expression is highly unlikely. Likewise, a PPV of 85.7% indicates that if the EB MIC is ≥25 μg/ml, there is a reasonable chance that the increased expression of one or more pump genes is present. However, none of these values is good enough to recommend EB MIC testing as a surrogate for gene expression analyses.

EB MIC and efflux. Table 4 illustrates the relationship between the EB MIC and an efflux phenotype. The sensitivity, specificity, PPV, and NPV calculated from these data are 95.4, 98.8, 98.8, and 95.2%, respectively. False positives and false negatives were very few in number. The EB MIC was ≥25 μg/ml for 83 strains that also had an efflux phenotype (true positives), and the EB efflux of these strains was 45.6% ± 14.1%; EB MICs were ≤12.5 μg/ml for 80 strains that had no efflux phenotype (true negatives), and the EB efflux was 11.0% ± 4.4% ($P < 0.001$, Mann-Whitney rank-sum test). The efflux phenotype was inhibited by reserpine in most instances,

TABLE 4. EB MIC (in μg/ml) and the presence of an efflux phenotype[a]

Efflux phenotype	MIC_{50}/MIC_{90} (range)	No. (%) of strains by EB MIC		Total
		≥25	≤12.5	
Positive	25/50 (6.25–≥100)	83 (95.4; TP)	4 (4.6; FN)	87
Negative	12.5/12.5 (3.13–25)	1 (1.2; FP)	80 (98.8; TN)	81
Total		84	84	168

[a] TP, true positive; FP, false positive; TN, true negative; FN, false negative.

with a reduction in efflux of 51.6% ± 28.4% observed. Of special interest were the 12 strains with an efflux phenotype that did not have the increased expression of any MDR pump gene (false-positive strains) (Table 3). Efflux in nine strains was inhibited by reserpine at least 20% (range, 26 to 75%); efflux in the remaining three was less affected, but an effect was still present. Efflux in these strains is the result of an as-yet uncharacterized mechanism, but in most instances it remains sensitive to the inhibitory effect of reserpine.

Our data indicate that if the EB MIC for a strain of *S. aureus* is ≥25 μg/ml, then that strain has an efflux phenotype. MIC determinations and efflux assays are functional in nature, and the impact of a potential disconnect between transcription and translation that adversely affects the ability of the EB MIC to predict increased efflux pump gene expression, which is not a functional assay, does not apply.

Combining MIC, efflux assay, and gene expression data, 71 strains were identified for which the EB MIC was ≥25 μg/ml, were efflux proficient, and had the increased expression of at least one MDR efflux pump gene. The overexpression of *norA* with or without the increased expression of other genes was present in 62/71 (87.3%). This may have clinical relevance in that hydrophilic fluoroquinolones are good NorA substrates (13, 14). Knowledge that a strain is efflux proficient may alter antimicrobial agent selection by the avoidance of fluoroquinolone use.

Concluding remarks. Our data establish that, in *S. aureus*, a simple microtiter EB MIC determination is highly sensitive and specific in identifying efflux-proficient strains. Using this approach, epidemiologic data relating to efflux can be generated for large numbers of strains quickly and inexpensively. No specialized equipment or supplies are necessary, and results are available within 1 day. The primary drawback to EB MIC screening is that it does not define the pump(s) involved. If such data are required, qRT-PCR can be used to provide it. The initial performance of EB MIC screening will reduce the number of strains requiring analysis by qRT-PCR, significantly reducing expenses. A potential drawback is the one-dilution difference between effluxing and noneffluxing strains and the commonly accepted 2-fold day-to-day variability in microtiter MIC testing results. This issue can be limited by the performance of a large number of replicates, such as the quadruplicate testing we employed. For the 87 effluxing strains we identified (true positives and false negatives) (Table 4), observed MICs were ≤12.5, 25, and ≥50 μg/ml (4, 60, and 23 strains, respectively). If the MIC cutoff for an effluxing strain were increased to 50 μg/ml, the majority of true effluxing strains would be missed, and thus it is not recommended.

The predominance of increased *norA* expression among efflux-proficient strains establishes NorA as the most clinically relevant MDR efflux pump among geographically diverse clinical strains of *S. aureus*. In addition to reducing the susceptibility of *S. aureus* to select fluoroquinolones, NorA also provides some protection of the organism to quaternary biocide agents. Such agents are commonly used as disinfectants in health care settings, and increased *norA* expression may provide a survival advantage to environmental strains. EB MIC screening can be used to determine the prevalence of efflux proficiency among such strains and whether or not repeated exposure to these biocides selects for this phenotype.

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